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We show that the metalloproteinase matrilysin dramatically accelerates the development of neu-induced mammary tumors by decreasing the time to tumor onset and increasing the percent of animals developing tumors. In addition, MMTV-matrilysin transgenic mice develop preneoplastic alveolar nodules in multiparous females. We speculated that the mechanism of tumor acceleration was via the induction of an EGF receptor signalling pathway, but an analysis of the interaction and phosphorylation of erbB family members demonstrated no evidence for activation of these pathways. Using human breast cancer cell lines, we demonstrated that the expression of matrilysin in MDA-MB-468 cells requires the presence of an EGF signalling pathway. However, in addition to the positive activation of matrilysin, the loss of a negative repressor of matrilysin expression is also required for constitutive matrilysin expression as demonstrated by fusion with the immortal breast epithelial cell line HBL 100. Determining 1) the mechanism of matrilysin acceleration of mammary tumorigenesis and 2) characterization of the matrilysin repressor will be pursued in future studies.

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FOREWORD

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INTRODUCTION

The matrix-degrading metalloproteinases (MMPs) have been implicated in tumor progression, invasion, and metastasis by virtue of their ability to degrade extracellular matrices. The genes for these enzymes are regulated both positively and negatively by growth factors. Our initial hypothesis was that the action of growth factors on mammary neoplasia and tumor progression is mediated, at least in part, by regulating MMP levels. The specific aims of the project were: 1) Examine the expression of matrilysin, stromelysin-1, and stromelysin-3 in transgenic mice overexpressing TGFα and/or TGFβ in the mammary gland under the control of the MMTV promoter. 2) Characterize MMTV-matrilysin transgenic mice for effects of the transgene on mammary gland development and DMBA-induced tumorigenicity. 3) Examine the effects of inhibiting matrilysin levels in mammary tumorigenesis using MMTV-TIMP mice and matrilysin null mice. 4) Examine the effect of MMP alterations and TGFb on tumorigenicity in MMTV-neu transgenic mice.

The first annual report focused on the generation of transgenic mice expressing wildtype, activated, and inactive matrilysin in the mammary gland (Specific aim 2). A manuscript describing the results of these studies has been submitted for publication (manuscript attached). The previous annual report described the preliminary results of experiments in which an enhancing effect of matrilysin was observed on neu-induced tumorigenesis in MMTV-neu/MAT bigenic mice (specific aim 4). This work has been expanded by the analysis of additional animals and the mammary gland of multiparous females, and the results are summarized below. We described the scientific rationale for a change in focus in the previous Progress Report to pursue the following possibilities: 1) matrilysin acts to accelerate mammary tumorigenesis via a mechanism that involves the EGF/erbB pathway, and 2) the effects of TGFα on tumorigenesis requires the induction of matrilysin. The results of these studies and a Statement of Work for the next set of studies proposed based on the results of these experiments follows.

PROGRESS:

Induction of Mammary Tumors in the MMTV-MAT Transgenics.

To investigate a potential role for MAT in mammary tumorigenesis, we induced mammary tumors in MMTV-MAT transgenic mice by mating them with MMTV-Neu animals. MAT/Neu, MAT, and Neu transgenic offspring were palpated weekly to determine the onset of mammary tumors. Bigenic MAT/Neu animals developed mammary tumors with a morphological and histological appearance similar to those previously reported in Neu single transgenic animals (Fig. 1A and B). Histological examination of lung tissue from these affected animals revealed the presence of multiple nodular lesions lodged in pulmonary vessels (Fig. 1C). These lesions were verified as metastases originated from mammary tumors by the presence of b-casein immunoreactivity (Guy et al, 1992, data not shown).

MMTV-MAT female transgenic mice (line #3) express detectable matrilysin protein throughout the epithelium of developing (weeks 6-14) and adult

mammary glands (see attached manuscript). The presence of the protein produced from the MAT transgene in the MAT/Neu mammary tumors was confirmed using an anti-MAT antibody that reacts with human, but not mouse MAT. The MAT protein product was detected in isolated groups of cells lying at the periphery of MMTV-MAT/Neu mammary tumors, and not in tumors derived from MMTV-Neu only transgenic mice (Fig. 1D and data not shown). Murine MAT mRNA was undetectable in approximately six samples of MMTV-Neu tumors analyzed (data not shown).

To test the hypothesis that overexpression of MAT increases the metastatic ability of mammary tumor cells, we determined the percent of double transgenic (MAT/Neu) and single transgenic (Neu) animals with secondary lung metastases. Eighty percent (12 of 15) of the MMTV-Neu animals developed lung metastases, while 91% (10 of 11) of the MMTV-MAT/Neu double transgenic animals developed lung metastases. Thus, the overexpression of MAT in the MMTV-Neu animals resulted in a small, but not statistically significant, increase in the metastatic ability of the mammary tumor cells.

Although the MAT-expressing mice showed no significant alterations in tumor metastasis, we observed a dramatic acceleration in tumor onset in MMTV-MAT/Neu mice compared to the MMTV-Neu control animals. Fifty percent of female bigenic animals developed mammary tumors by approximately 27 weeks, while 50% of single transgenic MMTV-Neu animals developed mammary tumors by approximately 40 weeks ($p<0.00001$ by a log-rank test, Fig. 2). In addition, 100% of the MAT/Neu double transgenic females formed mammary tumors by 40 weeks of age, whereas 20% of the Neu females were still tumor-free by 60 weeks of age (Fig. 2). Thus, the overexpression of MAT in Neu-expressing mammary glands enhanced tumorgenesis by increasing the frequency of tumor development and shortening the time of tumor onset by an average of 13 weeks.

Mammary tumor growth was monitored weekly by measuring the tumors with a caliper in two dimensions. The average doubling time of the MMTV-Neu tumors (14.0 ± 6.5 days) was not significantly different than the MMTV-MAT/Neu tumors (15.5 ± 6.7 days). These data indicate that although the double transgenic mice developed mammary tumors earlier, the rate of growth for each tumor once established was similar between the two groups of animals.

The overexpression of the MAT transgene in MMTV-MAT single transgenic female mice does not produce any observable morphological changes during mammary gland development (See manuscript attached). Careful examination, however, of mammary whole mounts showed that 50% (4/8) of aged, multiparous MAT wildtype line #3 transgenic females contained abnormal structures in the mammary glands (Fig. 3A), while control multiparous non-transgenic mammary glands were devoid of such structures (0/6, Fig. 3B). These distinctive focal areas of epithelial hyperplasia have been previously termed hyperplastic alveolar nodules (HANs), and are considered to be premalignant precursors that are prone to develop into mammary carcinomas (Daniel and Silberstein, 1987; Cardiff, 1984). Previous studies suggest that the HAN is probably derived from a single cell; with each HAN representing a clonal

population of cells (Cardiff, 1984). However, it has also been demonstrated that an individual HAN population can undergo further genetic changes that result in a biologically heterogeneous population of hyperplastic cells (Cardiff, 1984). For example, when a single HAN is divided and each portion transplanted into a separate fat pad, the resulting outgrowths are morphologically and biologically diverse, demonstrating the pluripotent nature of the HAN population. The appearance of HANs in the MMTV-MAT mammary glands and not in age-matched and pregnancy-matched nontransgenic controls suggests that the overexpression of MAT predisposes to the formation of these preneoplastic lesions.

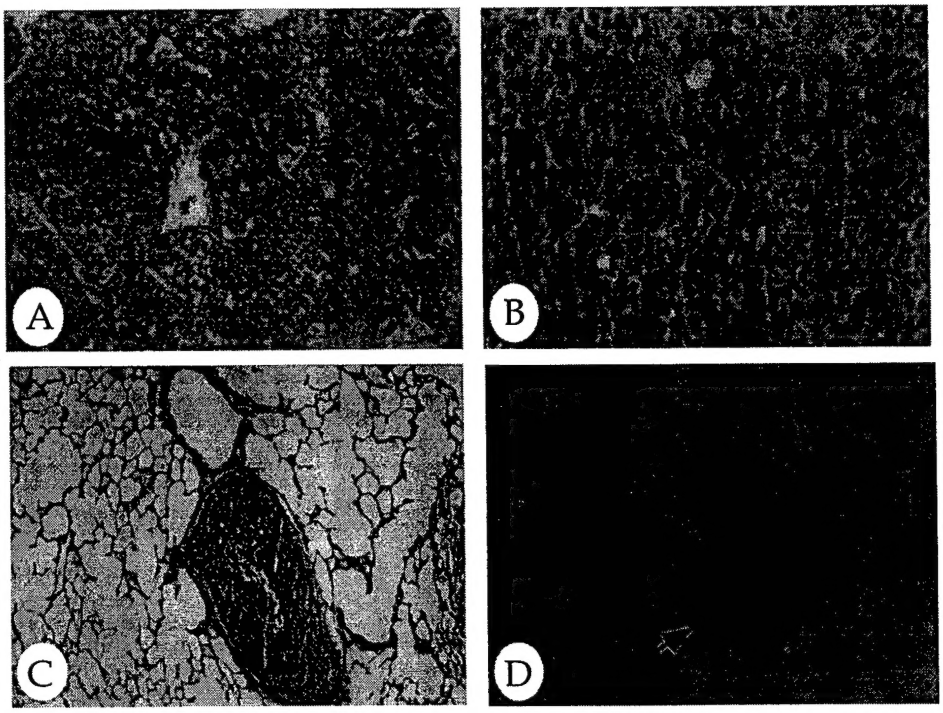

Figure 1. Histological appearance and MAT expression in MMTV-Mat/Neu mammary tumors. A typical hematoxylin and eosin-stained section of a mammary tumor from a MMTV-MAT/neu animal. A) 16X magnification and B) 50X magnification. C) A lung metastasis (LM) from a MMTV-MAT/neu animal with mammary tumors (11X magnification). D. Immunolocalizatoin of MAT in the MMTV-MAT/neu mammary tumors to the periphery and border of the tumor (arrows). T = primary mammary tumor (50X magnification).

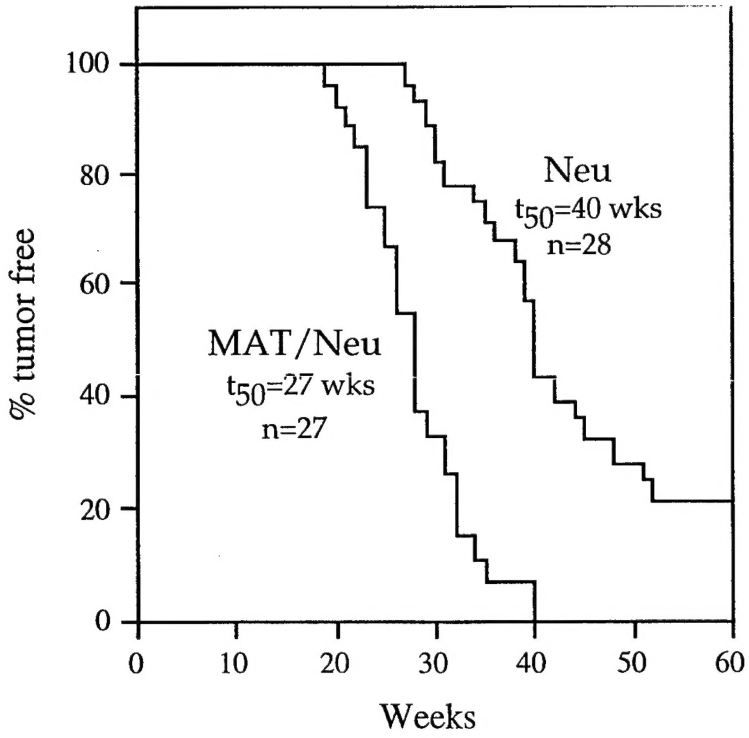

Figure 2. Mammary tumor onset in MMTV-MAT/neu and MMTV-neu transgenic animals. Analysis of the time to development of palpable mammary tumors in double and single transgenic females. The difference is statistically different by log-rank test ($P<0.00001$).

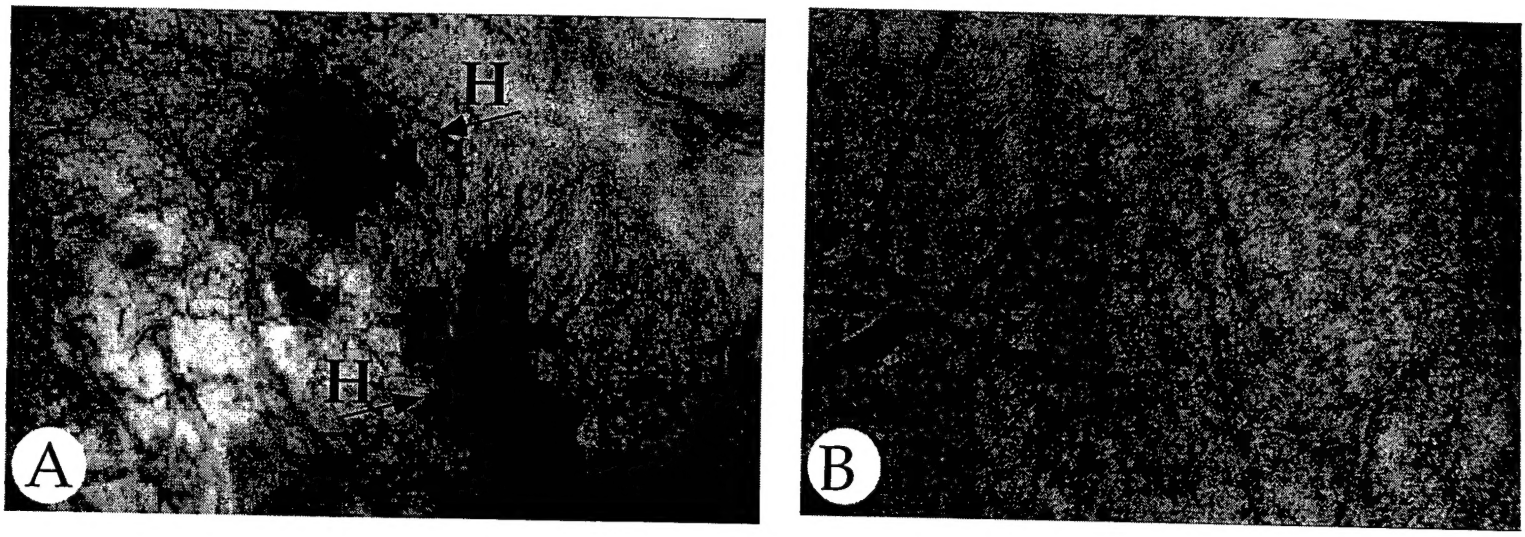

Figure 3. Multiparous mammary gland phentype in the MMTV-MAT transgenic mice. Whole mount staining of representative inguinal mammary glands from multiparous transgenic (A) and nontransgenic (B) mice. Note the appareance of HANs (H).

Function of Growth Factor Receptors in the Induction of Mammary Tumors.

The dramatic effect of MAT on mammary tumorigenesis raised the question of the mechanism by which metalloproteinase activity accelerates Neu-induced mammary tumor formation. The development of mammary tumors in the MMTV-Neu transgenic animals is caused by various deletions within the Neu transgene, which constitutively activate the Neu signal transduction pathway (Siegel et al., 1994). Only the mammary tumor cells developed deletions within the Neu transgene, while uninvolved mammary glands in the same animals do not have these Neu associated alterations (Siegel et al., 1994). These data imply that constitutive activation of the Neu signal transduction pathway is responsible for the induction of mammary tumors. Neu signal transduction is dependent on heterodimerization with ligand-binding erbB receptor family members, since Neu does not directly bind ligand (reviewed in Earp et al, 1995). Members of the EGF family of ligands, including EGF, TGFα, HB-EGF, amphiregulin, and betacellulin, can transmit this signal through their association with ErbB1, the EGF receptor. Similarly, ligation of the Heregulin family of ligands with ErbB3 or ErbB4 can also transmit a mitogenic signal. The role of these signal transduction pathways in Neu-induced mammary tumorigenesis has been substantiated by studies examining the rate of tumor onset in MMTV-TGFa/Neu animals (Muller et al., 1996). The MMTV-TGFα/Neu animals develop mammary tumors earlier than the MMTV-Neu animals, and the spontaneously derived tumors in the MMTV-TGFα/Neu animals do not contain deletions within the Neu transgene that are associated with tumor onset in the MMTV-Neu mice. These data suggest that overexpression of TGFα in the MMTV-Neu animals provides excess ligand for the constitutive activation of the Neu signal transduction pathway, thereby negating the need for activating deletions (Muller et al., 1996).

In our studies, the MMTV-MAT/Neu animals developed mammary tumors earlier than the MMTV-Neu transgenics, suggesting that a mechanism similar to that observed in the MMTV-TGFα mice may be in operation. We hypothesized that MAT activity could result in an increase in the availability of soluble ErbB receptor ligands, either through cleavage of membrane precursors or release of ligand from matrix components. The processing of TGFα (Massague and Pandiella, 1993) and HB-EGF Lazerein et al, 1993) to their soluble forms is inhibited by synthetic inhibitors of metalloproteinases, providing support for this hypothesis. In addition, several EGFR ligands, including HB-EGF and amphiregulin, have high affinity for proteoglycans (Cook et al, 1995), and MAT is a potent proteoglycanase (Murphy et al, 1991). To test this hypothesis, we analyzed the ability of Neu to heterodimerize and activate other erbB family members in MAT/Neu and Neu mammary tumors.

Antibodies specific to ErbB-1 (the epidermal growth factor receptor, EGFR), ErbB-3 and ErbB-4 were used to immunoprecipitate these receptors from mammary tumor protein lysates, either as complexes or isolated molecules. The

presence of Neu within preexisting cellular complexes was then analyzed by western blotting. Immunoprecipitation with anti-EGFR and subsequent blotting for Neu revealed that Neu protein co-immunoprecipitated with the EGFR (Figure 4A for representative samples). In addition, Neu was shown to co-immunoprecipitate with ErbB-3 and ErbB-4 in the same mammary tumor protein lysates (data not shown). There was no discernible difference in the association of Neu with other family member receptors in the MMTV-MAT/Neu mammary tumors compared to the MMTV-Neu tumors.

To determine if signalling through ErbB receptors occurred, phosphotyrosine levels (p-Tyr) of these proteins was analyzed by immunoprecipitation with anti-receptor antibody and western blotting with anti-p-Tyr (Fig. 4B and C for representative samples). The EGFR was detected and was phosphorylated at moderate levels in both the MAT/Neu and Neu mammary tumor extracts (Fig. 4B). High levels of Neu were also found in mammary tumors and were associated with high levels of p-Tyr in both sets of tumors (Fig. 4C). ErbB-3 and ErbB-4 were detected within the mammary tumor extracts, but were associated with very low or undetectable levels of p-Tyr (data not shown). These data illustrate that the EGFR and Neu receptor were the only ErbB family members that were activated at relatively consistent levels within the mammary tumor extracts. Importantly, the level of activation of the ErbB receptors was similar between the MMTV-MAT/Neu tumors and the MMTV-Neu tumors, suggesting that MAT has no effect on the levels of ErbB receptor signalling in fully-developed mammary tumors.

Constitutive activation of the Neu receptor by small deletions in the cytoplasmic domain has been demonstrated to contribute to the development of spontaneous mammary gland tumors observed in the MMTV-Neu animals (Siegel et al., 1994). Examination of the Neu transgene by RNAse protection revealed deletions in 67% (4/6) of our MMTV-MAT/Neu mammary tumor samples, while uninvolved mammary glands from these same animals lacked any Neu alterations (data not shown). The development of mammary tumors therefore corresponds to mutations in the *c-neu* transgene in MMTV-MAT/Neu mice similar to that observed in MMTV-Neu mice.

Phosphorylation and activation of the ErbB receptors could also presumably occur before the development of the mammary tumors. Mammary glands from MAT/Neu and Neu virgin animals at 25 - 30 weeks of age that were free of mammary tumors were processed and protein extracts examined for the presence of ErbB receptors and levels of p-Tyr. These data revealed little difference between the expression levels of the ErbB receptors, or their levels of p-Tyr between the MAT/Neu and the Neu alone virgin, tumor free mammary glands (data not shown).

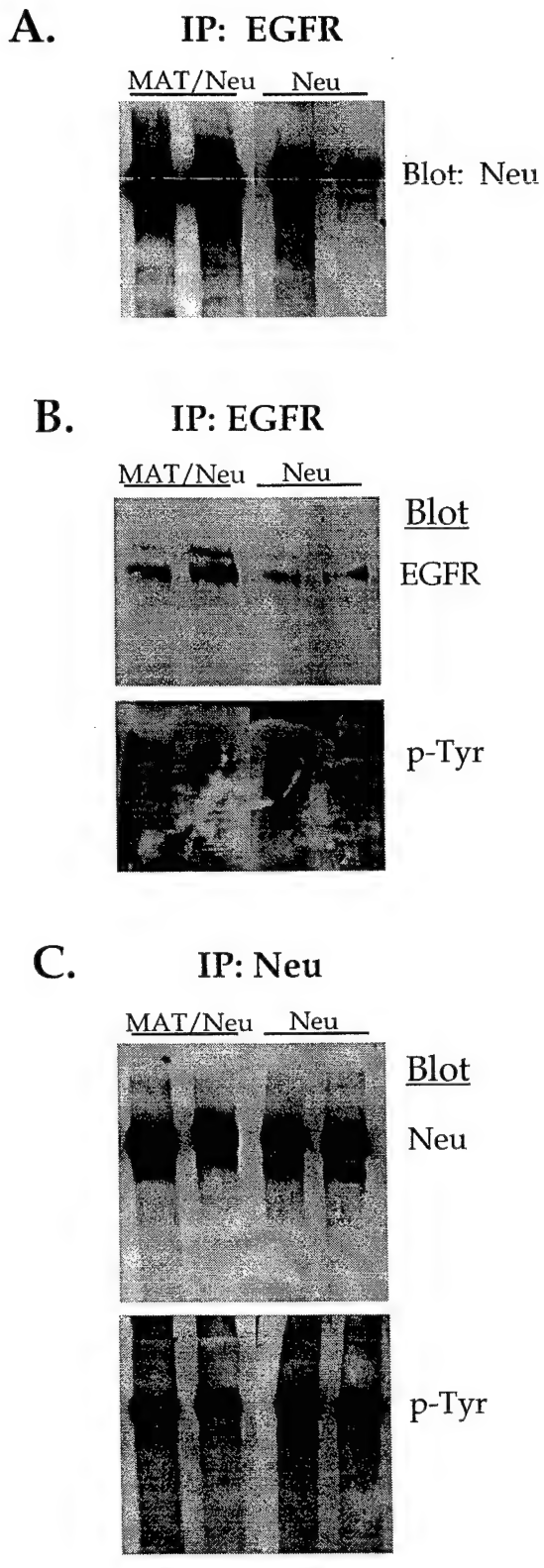

Figure 4. ErbB receptor expression in the MMTV-MAT/neu and MMTV-MAT mammary tumors. A) Co-immunoprecipitation of the ErbB family of receptors in MMTV-MAT/neu and MMTV-neu mammary tumor extracts. Immunoprecipitation of the EGFR, followed by western blot for the Neu protein. B and C) Immunoprecipitation and western blot analysis of ErbB receptors in mammary tumor extracts from MMTV-MAT/neu and MMTV-neu animals. ERGR (B) and Neu (C) was specifically immunoprecipitated from 300ug of mammary tumor extracts followed by western blotting for tyrosine phosphorylation with a p-Tyr antibody (p-Tyr) or for the presence of the immunoprecipitated receptor to control for the loading of protein. Five to eight mammary tumor samples were assayed and two representative samples are shown.

Regulation of matrilysin in human breast cells.

In our previous Progress Report we discussed data that indicated that the expression of matrilysin in human breast cancer MDA-MB-468 cells was dependent upon a positive signal through the EGF receptor. If this was the case, it was surmized that the induction of matrilysin in breast cancer might be through a "positive" mechanism requiring the activation of an oncogenic pathway. To test this hypothesis somatic hybrids between non-matrilysin expressing HBL 100 cells, an immortal cell line derived from human breast milk, and MDA-MB-468 cells were generated. If activation of the EGF receptor signalling pathway was sufficient for matrilysin expression one would surmise that expression of this gene would be a dominant trait in somatic hybrids.

Somatic hybrids were generated between MDA-MB-468 cells and HBL 100 cells through the transfection of different dominant selectable genetic markers into each of the parental cell populations (hygromycin phosphotransferase into HBL 100 cells conferring resistance to hygromycin B; neomycin phosphotransferase conferring resistance to geneticin (G418) into MDA-MB-468 cells), poly-ethyleneglycol mediated cells fusion and subsequent selection for doubly resistant cells. To confirm the hybrid nature of the cells flow cytometric DNA analysis of somatic hybrids was utilized. While the HBL100-hyg cells and the MDA-MB-468-neo cells showed a cell cycle profile similar to each other, the hybrid cells displayed a G1 cell cycle phase peak which corresponded to the G2 phase peak of the parental cells (data not shown). This result coupled with the fact that the selected cells were resistant to both hygromycin B and G418 selection indicated that the hybrid cells were in fact comprised of two genomes, one from each parental cell population.

The HBL 100-hyg, MDA-MB-468-neo and hybrid cells were injected subcutaneously into nude mice. No growth was evident from the HBL 100-hyg cells (0/5 mice), the MDA-MB-468-neo cells demonstrated rapid growth when injected into nude mice subcutaneously. These cells also showed a 100% tumor take at the number of cells inoculated. The hybrid cells on the other hand showed a markedly delayed tumor onset in 3 of 5 animals. This result is characteristic of somatic hybrid cells and reflect the "tumor suppressive" influence of the non-malignant cells.

Parental cells and hybrid cells were analysed for the expression of matrilysin by northern blot analysis. No detectable matrilysin transcript was found associated with the HBL 100-hyg cells, while MDA-MB-468-neo cells maintain the same pattern of expression (Figure 5). When the hybrid cells were analysed, however, it was found that they demonstrated the HBL 100-hyg phenotype, that is, these cells did not express matrilysin under any of the conditions tested.

Our results suggest that matrilysin expression in MDA-MB-468 cells requires EGF receptor signalling and that a factor conferred by somatic fusion with HBL 100 cells represses matrilysin expression. These observations raised the possibility that the mechanism for repression of matrilysin expression in the hybrid cells might be through an influence that the HBL 100 component has on the EGF signalling pathway.

Analysis of EGF receptor levels by flow cytometry revealed that the MDA-MB-468 cells express the EGF receptor at a higher level than the HBL 100 cells (Figure 6). In the same experiment, hybrid cells were found to express EGF receptor levels similar to that seen in the MDA-MB-468 cells.

Northern blot analysis was carried out to determine if downstream consequences of EGF receptor mediated signalling were intact in the HBL 100 and hybrid cells. Cells were treated with EGF and analysed for the expression of c-*fos* RNA after 30 and 90 minutes (Figure 7). c-*fos* RNA was found to be elevated in both HBL 100 and MDA-MB-468 cells at 30 minutes and returned to basal levels after 90 minutes. A robust induction of c-*fos*, similar to that seen in MDA-MB-468 cells was observed in the hybrid cells demonstrating that the EGF receptor mediated signalling pathway, at least to the level of c-*fos* induction, was intact in the hybrid cells.

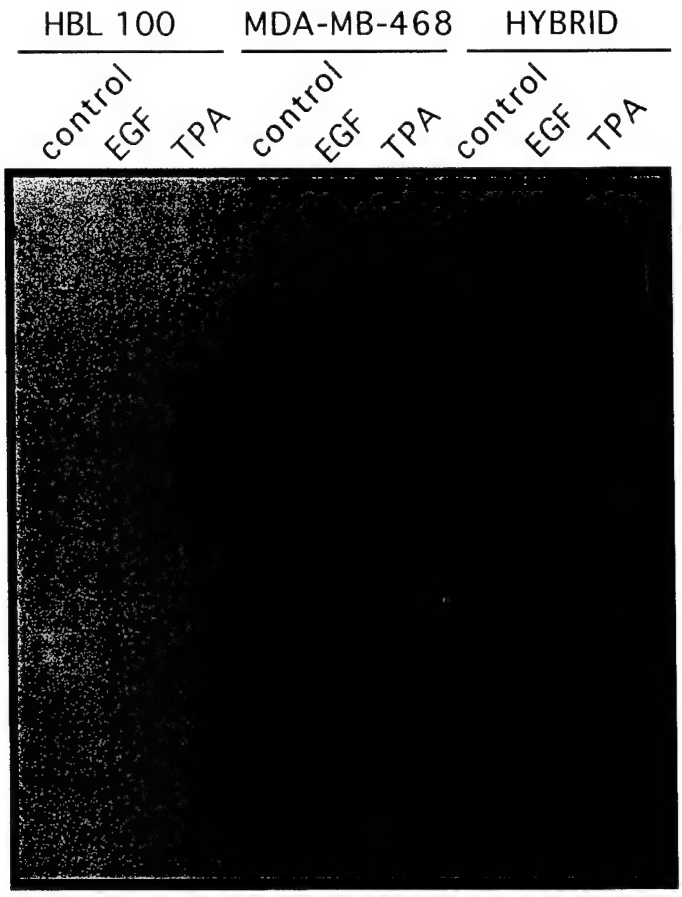

Figure 5. Northern blot analysis of constitutive and inducible matrilysin expression in HBL 100-hyg, MDA-MB-468-neo and hybrid cells. HBL 100-hyg, MDA-MB-468-neo and hybrid cells were grown under serum free conditions either untreated (lanes marked control) or supplemented with 50 ng/ml EGF (lanes marked EGF) or 100 ng/ml TPA (lanes marked TPA). **Panel A)** Hybridization of the blot with a probe to matrilysin. **Panel B)** Ethidium bromide stained gel to demonstrate equal loading of total RNA.

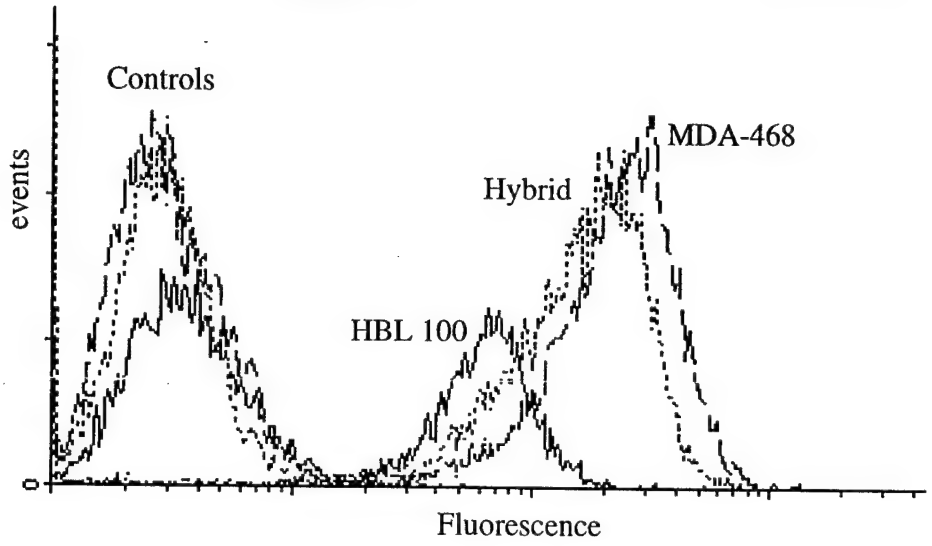

Figure 6. Frequency histograms derived from FACS analysis of cellular expression of EGF receptor. HBL 100-hyg, MDA-MB-468-neo and hybrid cells were exposed to an antibody raised against the human EGF receptor (open region) or a control IgG (filled region), washed, subsequently exposed to an FITC conjugated secondary antibody and then analysed by flow cytometry. The top panel shows the histogram profile for HBL 100-hyg cells, the middle panel shows the histogram profile for the MDA-MB-468-neo cells while the lower panel shows the histogram profile for the hybrid cells.

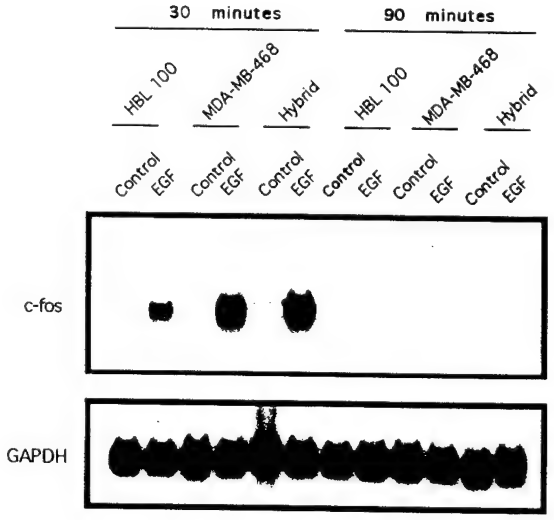

Figure 7. *Northern blot analysis of c-fos RNA expression in HBL 100-hyg, MDA-MB-468-neo and hybrid cells in response to stimulation with EGF.* HBL 100-hyg, MDA-MB-468-neo and hybrid cells were grown under serum free conditions either untreated (lanes marked control) or supplemented with 50 ng/ml EGF (lanes marked EGF) and subsequently analysed for c-fos RNA expression after 30 and 90 minutes. The same blot was stripped and subsequently reprobed for GAPDH RNA expression to normalize for RNA loading.

CONCLUSIONS:

The results from the MMTV-matrilysin transgenic mice demonstrate that matrilysin is sufficient to induce preneoplastic HANs and it accelerates the formation of neu-induced mammary tumors. We proposed that this activity was a result of activation of an erbB pathway, but immunoprecipitation results did not support an increase in erbB signalling as measured by phosphorylation of erbB receptors. As a result, we propose to continue these studies by examining signalling through the insulin-like growth factor (IGF) receptor, as well as by measuring levels of mammary epithelial cell proliferation and apoptosis in MMTV-matrilysin mammary glands as described in the Statement of Work.

We also proposed that matrilysin is induced in breast cancer as a result of signalling through the EGF/erbB pathway. This hypothesis was tested using the human breast epithelial cell lines MDA-MB-468 and HBL 100. Our results demonstrated that a positive signal through the EGFR was required for the expression of matrilysin, but also demonstrated that the loss of a repressor of matrilysin was also required as determined by cell fusion studies. This repressor appears to act directly on matrilysin expression, and does not interfere with EGF signalling to c-*fos*. We propose to pursue the characterization of the negative regulator of matrilysin expression since this regulatory may represent a tumor suppressor that is lost in the progression of human breast cancer.

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REVISED STATEMENT OF WORK
1997-1998

Mechanism of matrilysin enhancement of mammary tumors:

1. Examine alternations in the activation of the IGF receptor in MMTV-matrilysin transgenic mice compared to nontransgenic control mammary glands using immunoprecipitation and western blotting techniques.

2. Examine the proliferation and apoptotic rates of mammary epithelial cells in MMTV-matrilysin compared to nontransgenic control mammary glands. Techniques to be used include immunostaining for proliferation-related antigens and terminal transferase labeling of fragmented DNA.

Regulation of matrilysin expression in mammary tumors:

3. Determine if matrilysin gene expression is inhibited by the negative regulator in HBL100 cells by nuclear run-on assays and transfection of matrilysin promoter/reporter constructs.

4. If matrilysin gene expression is transcriptionally repressed, map the cis-acting elements responsible for this repression to provide insights into the molecular nature of the repressor.

Overexpression of the matrix metalloproteinase matrilysin results in premature mammary gland differentiation and male infertility

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Running Title: Overexpression of matrilysin alters reproductive function

Abbreviations: MMTV-LTR, murine mammary tumor virus long terminal repeat; MMP, matrix metalloproteinase; MAT, matrilysin

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ABSTRACT

To examine the role of matrilysin (MAT), an epithelial cell-specific matrix metalloproteinase, in the normal development and function of reproductive tissues, we generated transgenic animals that overexpress MAT in several reproductive organs. Three distinct forms of human MAT (wildtype, active and inactive) were placed under the control of the MMTV promoter/enhancer. Although wildtype, active and inactive forms of the human MAT protein could be produced in an in vitro culture system, mutations of the MAT cDNA significantly decreased the efficiency with which the MAT protein was produced in vivo. Therefore, animals carrying the wildtype MAT transgene which expressed high levels of human MAT in vivo were further examined. Mammary glands from female transgenic animals were morphologically normal throughout mammary development, but displayed an increased ability to produce β-casein protein in virgin animals. In addition, beginning at approximately 8 months of age, the testes of male transgenic animals became disorganized with apparent disintegration of interstitial tissue that normally surrounds the seminiferous tubules. The disruption of testis morphology was concurrent with the onset of infertility. These results suggest that overexpression of the matrix-degrading enzyme, MAT, alters the integrity of the extracellular matrix and thereby induces cellular differentiation and cellular destruction in a tissue-specific manner.

INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of extracellular proteases thought to be responsible for normal matrix remodeling and pathological tissue destruction by virtue of their ability to catabolize extracellular matrix components (Birkedael-Hansen *et al*, 1993; Hulboy *et al*, 1997 for review). The expression pattern of MMPs in normal tissues suggests that they are particularly involved in the remodeling associated with reproductive processes, including menstruation, trophoblast invasion, mammary gland morphogenesis, and involution of the uterus, mammary gland and prostate (Hulboy *et al*, 1997). Studies with natural and synthetic inhibitors of MMPs (Marbaix *et al*, 1996; Talhouk *et al*, 1992; Butler *et al*, 1991; Brannstrometal *et al*, 1988), and recent experiments which utilized genetically altered mice that express altered MMP substrates (Liu *et al*, 1995) or lack specific MMP family members (Rudolph-Owen *et al*, 1997), have confirmed the importance of MMPs in reproductive processes in several systems.

Sixteen MMP family members have been described (reviewed in Hulboy *et al*, 1997; see also Cossins *et al*, 1996; Puente *et al*, 1996). All MMPs described have three essential domains: a signal sequence or pre domain to direct secretion from the cell, a pro sequence to maintain latency, and a catalytic domain containing the critical zinc-binding site. Matrilysin (MAT, MMP-7, pump-1, uterine metalloproteinase, EC #3.4.24.23) is unique in that it contains only these minimal domains. All other MMP family members have an additional hemopexin/vitronectin-like domain which is connected to the catalytic domain by a variable hinge region (Birkedael-Hansen *et al*, 1993 for review). This, and other domains found in specific MMP family members, generate diversity by modifying properties such as substrate specificity, interaction with endogenous inhibitors of metalloproteinases, intracellular activation, and cell-surface localization (Powell and Matrisian, 1996 for review).

Matrilysin is considered a member of the stromelysin subfamily of MMPs. The stromelysins, including stromelysin-1 (STR-1, MMP-3, EC #3.4.24.17), stromelysin-2 (STR-2, MMP-10, EC #3.4.24.22), and MAT, can degrade a broad range of substrates such as fibronectin, proteoglycans, and denatured and basement membrane collagens. Subtle differences in substrate specificity within this subfamily have been observed. For example, although extracellular matrix (ECM) proteoglycans are substrates for MAT, STR-1, and STR-2, MAT can degrade elastin (Murphy *et al.*, 1991; Imai *et al.*, 1995), entactin (Sires *et al*, 1993) and tenascin (Imai *et al*, 1994; Siri *et al*, 1995) more efficiently than the other stromelysins. MAT is also distinct from most MMP family members in that it is expressed primarily by normal and malignant glandular epithelial cells. MAT is expressed in the epithelial cells of the cycling human endometrium, small intestinal crypts, and postpartum and cycling mouse uterus, as well as epithelial tumors of the gastrointestinal tract, prostate, and breast (Wilson and Matrisian, 1996 for review). The stromelysins and most other MMP family members in contrast are expressed primarily in mesenchymal tissues, including endometrial stromal (Hulboy *et al*, 1997 for review) and stromal cells surrounding several tumor types (Powell and Matrisian, 1996 for review). The unique protein structure, altered substrate specificity, and uncommon localization patterns of MAT suggests that this MMP may have in vivo functions that are distinct from other stromelysins and MMP family members.

Since MMPs are expressed in a variety of reproductive organs and there are features that distinguish MAT from other stromelysins and MMP family members, we were interested in determining the effects of MAT overexpression in reproductive organs. Transgenic mice expressing wildtype, inactive, and constitutively active MAT under the control of the MMTV-LTR were generated and their affect on mammary gland development and male reproductive function was

assessed. The comparison of these transgenic mice with other MMP-expressing mice provides insights into the action of specific MMPs in reproductive processes.

MATERIALS AND METHODS

Plasmid Construction

The 1.1-kilobase full-length human MAT cDNA (pPump-1; Muller *et al*, 1988) was altered by oligonucleotide-directed mutagenesis. An active form of human MAT with a substitution of valine to glycine at amino acid 92 was generated with the oligonucleotide 5′-CCGGTGTGGTGGGCCCGACGTC-3′ and cloned into pKCR3 (Witty *et al*, 1994). An inactive form of human MAT with a substitution for glutamic acid to glutamine at amino acid 216 was generated with the oligonucleotide 5′-ATGGCCAAGTTGATGAGTTGC-3′ and also cloned into pKCR3. The resulting full-length human MAT cDNAs (wildtype, active and inactive, Figure 1B) were subcloned into the unique EcoRI site of the MMTV-LTR expression vector pMMTVEV (Matsui *et al*, 1990) to generate pMMTV-MAT, pMMTV-ActMAT, and pMMTV-InMAT, respectively (Figure 1A). The expression vector contains intron, splice sites and polyadenylation signals derived from the rabbit β-globin gene which increase the efficiency of expression (Breathnach and Harris, 1983).

Human MAT cDNA probes were generated by digesting full-length human MAT in pPump-1 (Muller *et al*, 1988) with EcoRI and XbaI to remove the poly(A)+ tail. The 1.1 kb human MAT fragment was then subcloned into pGEM7zf(+) to yield pG7pumpEX. For in situ hybridization, a 356-bp fragment of human MAT cDNA corresponding to positions +700 to + 1056 was amplified by PCR using the primers 5'-CGCGTCTAGACCTCTGATCCTAATGCAG-3' and 5'-CGCGAAGCTTGACATCTACGCGCACTG-3'. The human MAT fragment was subconed into pGEM7zf(+) to yield pG7-HmatUT, and linearized with HindIII or

XbaI to generate riboprobe templates for transcription using either T7 (antisense) or SP6 (sense) RNA polymerase, respectively.

Generation of MMTV-MAT Transgenic Mice

The three human MAT constructs, pMMTV-MAT, pMMTV-ActMAT, and pMMTV-InMAT, were purified by CsCl centrifugation and the Aat II/Bgl II fragment was then isolated by gel electrophoresis on low melting point agarose (SeaKem Agarose; FMC BioProducts, Rockland, ME) and purified using Gelase (Epicentre Technologies, Madison, WI). The purified fragments were subsequently injected into FVB/N fertilized eggs by the Vanderbilt Transgenic/ES Cell Shared Resource and transfered to pseudopregnant mothers as described (Hogan *et al*, 1995). Transgenic founders were identified by Southern blot analysis of EcoRI-digested genomic tail DNA using a random-primed (DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN) 1.1 kb EcoRI/XbaI fragment of human MAT from pG7pumpEX. The approximate copy number of founder transgenic animals was determined by adding 10pg (1 copy) or 100pg (10 copies) of MMTV-MAT to genomic DNA from a nontransgenic mouse and comparing relative intensity of hybridization. Transgenic lines were generated by mating founder animals with FVB/N nontransgenic mice.

Immunoprecipitation

The human breast cancer cell line Hs578t was transiently transfected with 10μg of pMMTV-MAT, pMMTV-ActMAT, or pMMTV-InMAT. The cells were allowed to recover overnight, placed in serum- and methionine-free media for 6 hours, and labeled with 100μCi of ^{35}S-methionine for 14-16 hours. The MMTV promoter was induced by the addition of 1μM of dexamethasone (Dex) to the culture media for 14-16 hours before collection of the conditioned medium. The wildtype, mutant, and

inactive MAT protein from 9 x 10^5 TCA precipitable counts was immunoprecipitated from ^{35}S-labelled conditioned medium using a polyclonal antibody raised against human MAT (McDonnell *et al.*, 1991), and separated on a SDS polyacrylamide gel. In addition, the MAT protein in the conditioned medium was activated by incubation with 1mM of the organic mercuride 4-aminophenyl-mercuric acetate (APMA) at 37°C for 30 minutes before electrophoresis.

Tissue Preparation

The right thoracic and inguinal mammary glands and other organs were removed and frozen in liquid nitrogen or on dry ice and stored at -70° C. Tissue was later homogenized in a guanidinium/acid phenol solution, and total RNA extracted as described by Chomczynski and Sacchi (1987). Poly(A)+ RNA was then isolated from total RNA over an oligo dT cellulose (Collaborative Biomedical Products, Bedford, MA) column or a latex bead-oligo dT column (Oligotex; Qiagen, Chatsworth, CA).

Left thoracic mammary glands were routinely fixed overnight in 4% paraformaldehyde and PBS for whole mount staining, while the left inguinal mammary glands and the testis and epididymis were fixed in paraformaldehyde and embedded in paraffin for subsequent sectioning.

Northern Analysis

Three to four micrograms of poly(A)+ RNA were electrophoretically separated on a 1% agarose-formaldehyde gel, transferred to nitrocellulose membrane (MSI, Westboro, MA), and UV cross-linked (Stratagene, LaJolla, CA). Blots were hybridized at 42°C under high-stringency conditions (50% formamide, 5X SSC, 1X PAF, 20mM NaPO4, 0.1% SDS, 50 µg/ml salmon sperm, and 4% dextran sulfate) using the radiolabeled, random-primed (DNA Labeling Kit; Boehringer Mannhein)

1.1 kb EcoRI/XbaI fragment of the human MAT cDNA, the 700 bp ApaI/HindIII fragment of the mouse MAT cDNA from plasmid pG7-mMATAH (Wilson *et al.*, 1995), or the cDNA for the endogenously expressed cyclophillin gene (1B15; Danielson *et al.*, 1988) to control for RNA loading. Washes were carried out at 50°C in 0.1X SSC and 0.1% SDS.

In Situ Hybridization

Paraffin-embedded paraformaldehyde-fixed tissue sections 5-7 μm in thickness were placed onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and analyzed for the human MAT transgene expression as previously described (McDonnell *et al*, 1991). The slides were prehybridized for 2-4 hours, after which ^{35}S-labeled riboprobes at 1.2 x 10^6 cpm/slide were added and hybridized overnight at 50°C. The slides were dipped in photographic emulsion (type NTB2; Kodak, Rochester, NY), exposed for 2- to 4-weeks at 4°C, developed, and counterstained with hematoxylin. Background hybridization was assessed using the sense probe for each transcript analyzed.

Whole Mount Analysis

Inguinal and/or thoracic mammary glands were removed and placed flat in plastic embedding cassettes (Fisher Scientific, Pittsburgh, PA), fixed in 4% paraformaldehyde in PBS overnight, transferred to 70% ethanol and stored at 4°C. The glands were defatted in 100% acetone and stained with iron hematoxylin (0.1% w/v hematoxylin, 0.1M FeCl3, 0.17M HCL in 95% EtOH) for 3 hours (Medina, 1973). Whole mounted glands were destained in 0.025M HCL in 50% ethanol, dehydrated to xylene, and stored in 100% methyl salicylate. Glands were viewed using a Nikon dissecting microscope (Southern Micro Instruments, Atlanta, GA).

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded sections were dewaxed, hydrated through graded ethanols, treated with 0.6% hydrogen peroxide in methanol (to destroy endogenous peroxidase activity), microwaved in 0.1M sodium citrate for 3 minutes and 45 seconds at high power to unmask the antigens and exposed to blocking solution (10mM Tris, pH 7.4, 100mM $MgCl_2$, 0.5% Tween-20, 1% w/v bovine serum albumin, and 5% w/v goat serum) for 1 hour. Sections were incubated overnight at 4°C in blocking solution with affinity-purified rabbit anti-human MAT antibody (1:1000 dilution; kindly provided by Dr. William Parks, Washington University School of Medicine, St. Louis, MO; Saarialho-Kere *et al.*, 1995), or control rabbit IgG (Sigma Immunochemicals, St. Louis, MO). The sections were washed in TBST buffer (150mM NaCl, 10mM Tris, pH 8.0, and 0.05% Tween-20), and incubated with biotinylated anti-goat IgG (1:5000; Vector Laboratories, Burlingame, CA) for at least 1 hour at room temperature. Labelled cells were visualized using an avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories) and TrueBlue peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Sections were then counterstained with Contrast Red.

Tissues were similarly processed but without microwave treatment with a rabbit antibody to mouse casein (1:5000 dilution; kindly provided by Dr. Charles Daniel, University of California at Santa Cruz; Robinson *et al.*, 1993), or to proliferating cell nuclear antigen (PCNA; 1:100 dilution; SIGMA, St. Louis, MO; Waseem and Lane, 1990).

To localize the transgene protein in the testis and epididymis, tissue was dissected and frozen in OCT medium (Fisher Scientific) and liquid nitrogen. Five µm sections were post-fixed in Bouin's fixative, then washed in PBS for 10 minutes. Slides were dipped in saturated $LiCO_3$ to eliminate picric acid, and washed again in

PBS. Sections were analyzed for the expression of human MAT protein as described above for paraformaldehyde-fixed tissues except without microwave treatment.

Analysis of Programmed Cell Death

Paraffin-embedded sections were analyzed for apoptotic cells using a modification of the TUNEL assay (Gavrieli *et al*, 1992). Tissues were deparaffinized, endogenous peroxidases quenched with 1% hydrogen peroxide in ethanol, and incubated in chloroform to remove lipids and reduce background levels of staining, as previously described (Witty *et al*, 1995a). Tissues were then dehydrated, washed with 1X TBS, and free 3'-OH DNA ends were labeled with biotin-conjugated deoxyribonucleotide triphosphate (Boehringer Mannheim) using terminal deoxynucleotidyl transferase (TdT) (GibcoBRL, Grand Island, NY), followed by a 1:5000 dilution of a horseradish-peroxidase-strepavidin conjugated antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA). Labelled cells were visualized with 1,2-diaminobenzidine and hydrogen peroxide, counterstained with hematoxylin, dehydrated through ethanols to xylene, and coverslipped under Permount.

RESULTS

Generation and Evaluation of MAT Expression Constructs

To determine the effects of ectopic expression of the metalloproteinase MAT on select reproductive organs, the human MAT cDNA was placed under the control of the MMTV-LTR in a vector designed to contain flanking, splice and polyadenylation sites to improve expression efficiency (Figure 1A). This expression vector has been demonstrated to be effective in directing transgene expression to the murine mammary gland, salivary gland, brain, testes, and epididymis (Matsui *et al*, 1990; Witty *et al*, 1995b). Three distinct expression constructs were generated to

produce wildtype, constitutively active, and inactive forms of the MAT protein (Figure 1B). The constitutively active MAT cDNA contains a valine to glycine substitution in the highly conserved sequence PRCGVPDV, which corresponds to amino acids 88-95 near the carboxyl terminal end of the pro-domain. Mutations in the rat stromelysin-1 sequence in this same region leads to variants which have a significantly increased tendency to spontaneously generate active STR-1 (Sanchez-Lopez *et al.*, 1988, Park *et al*, 1991). MAT protein produced by this construct therefore theoretically circumvents any dependence on exogenous factors for activation. The catalytically inactive MAT cDNA contains a glutamic acid to glutamine substitution at position 216 within the highly-conserved zinc binding domain. Similar mutations in the rat STR-1 cDNA result in protein that cannot be activated by organic mercurides to auto-proteolyze (Sanchez-Lopez *et al*, 1988). We reasoned that this construct would encode a MAT protein with three-dimensional structure that deviates only slightly from wildtype MAT but lacks proteolytic activity. This inactive mutant would provide a control to determine if observable effects could be attributed to the catalytic activity of this metalloproteinase.

To determine if the wildtype, active, and inactive human MAT transgenic constructs were functional in vitro, the human breast cancer cell line Hs578t was transfected with each MMTV-MAT expression vector and the conditioned medium analyzed for MAT protein by immunoprecipitation. Specific MAT immunoreactivity was not detected in the conditioned medium of untreated transfected cells, but was induced by the addition of the synthetic glucocorticoid dexamethasone (Dex), a known inducer of the MMTV promoter/enhancer (Ringold, 1983; Figure 1C, compare lanes A&B, C&D, E&F). The wildtype MAT cDNA produces a 28kDa protein corresponding to the latent zymogen (Figure 1C, lane B). In the presence of the organic mercuride 4-aminophenyl-mercuric acetate (APMA), which activates the cysteine switch of MMPs (Birkedael-Hansen *et al*, 1993 for

review), wildtype MAT protein was cleaved and converted to 19kDa, consistent with the removal of the pro domain and conversion to the mature, active catalytic form (Figure 1C, compare lanes B to G). Immunoprecipitation of the constitutively active human MAT protein demonstrated that the majority of the protein in the medium of transfected cells was present in the 19kDa active form, and the residual 28kDa protein could be completely converted to the activated form with APMA treatment (Figure 1C, lanes D and H). These data indicate that the mutation in the pro domain of MAT results in constitutive activation of the enzyme in the absence of exogenous activators, as was predicted from the results of similar mutations in rat STR-1 (Sanchez-Lopez *et al*, 1988; Park *et al*, 1991). In contrast, the inactive mutant protein was produced as the higher molecular weight proenzyme form and was not converted to the mature form by APMA treatment, indicating that a mutation at amino acid 216 in human MAT prevents the enzyme from autoproteolytic cleavage and thus represents an inactivating mutation (Figure 1C, compare lanes F and I).

Generation of MAT-Expressing Transgenic Mice

To test the effects of overexpressing the epithelium-specific matrix-degrading enzyme MAT in select reproductive tissues in vivo, the MMTV-MAT expression vectors producing wildtype, active, and inactive MAT protein were used to establish transgenic mouse lines by standard pronuclear injection techniques. Transgenic mice were identified by Southern blot analysis and founder mice harboring the transgene were mated to establish transgenic lines (Figure 2). At least two lines per construct were established with varying copy numbers to control for insertional variation. The resulting transgenic lines were identified by the founder animal number and will be referred to hereafter as MMTV-MAT (MMTV-wildtype-matrilysin), MMTV-ActMAT (MMTV-active-matrilysin), and MMTV-InMAT (MMTV-inactive-matrilysin).

Expression and Localization of the MAT Transgene in Mammary Epithelial Cells

The MMTV-LTR promoter/enhancer has been used extensively to drive the expression of transgenes in the mammary epithelium (Cardiff and Muller, 1993 for review). The MMTV-LTR promoter activity responds to endogenous steroid hormone levels in the murine mammary glands during development, pregnancy and lactation, as well as during the normal estrous cycle (Gunzburg and Salmons, 1992). MAT expression in the transgenic animals was analyzed by northern blot analysis of poly(A+) RNA from developing mammary glands of female mice harboring the wildtype, active, and inactive human MAT constructs. We observed considerable variability in the expression of the MAT transgene within and between the various transgenic lines, presumably due to hormonal fluctuations. For example, approximately 42% (8/19) of transgenic mammary glands examined from MMTV-MAT line #3 expressed human MAT wildtype mRNA at various stages of mammary development. No correlation of human MAT mRNA expression could be made for a particular time during mammary development or during a specific day of the estrous cycle (data not shown). However, when animals in which the MAT transgene was expressed were compared, human MAT expression appeared abundant between 6 and 17 weeks of age in the MMTV-MAT line #3 and #42, and was absent in nontransgenic littermate controls (Figure 3A and data not shown). MMTV-ActMAT line #1 and #22 also displayed detectable levels of MAT mRNA during mammary gland development, although in general MAT mRNA levels appeared lower than for MMTV-MAT mice (Figure 3B). In contrast, MAT mRNA appeared as a smear instead of a distinct band when isolated from the mammary glands of both the MMTV-InMAT line #2 and line #4 animals (Figure 3C). Several attempts were made to extract intact human MAT RNA from the mammary glands of the MMTV-InMAT transgenic lines, all of which proved to be futile. Endogenous mouse MAT mRNA was not detectable by northern blot analysis in the developing

mammary glands of either nontransgenic or MMTV-MAT transgenic mice (data not shown). However, we have previously shown by RT-PCR that low levels of mouse MAT are expressed in the adult mammary gland (Wilson *et al.*, 1995).

Immunohistochemistry was performed to localize the product of the MAT transgene in the mammary glands. Protein expression from the MMTV-MAT wildtype transgene was detected during several stages of mammary gland development (6 to 14 weeks), with staining localized to the cytoplasm of the epithelial cells of the mammary ducts (Figure 4A and data not shown). MAT protein was also detected in mammary tissue from the MMTV-ActMAT lines, but at relatively lower levels than the MMTV-MAT wildtype lines (Figure 4B). We detected no MAT immunoreactivity in mammary glands from the MMTV-InMAT animals (Figure 4C), suggesting that the mutation impairs the production of MAT protein in vivo. No immunoreactivity was detected in any mammary gland sections from nontransgenic littermate controls at various times of mammary development (Figure 4D for example). Because of the absence or low expression of MAT protein in the transgenic animals carrying the mutated human MAT cDNA constructs and the high MAT expression in the transgenic animals carrying the wildtype human MAT cDNA construct, we focused our attention in subsequent studies on those animals carrying the wildtype MAT transgene. In general, initial observations suggest that transgenic animals carrying the activated form of MAT showed similar phenotypes to wildtype MAT transgenics but to a lesser degree, while the inactive MAT transgenics were indistinguishable from nontransgenic controls.

Consequences of MAT Overexpression in the Mammary Glands

Previous studies have indicated that the expression of the MMP STR-1 in mammary epithelial cells results in disruption of the basement membrane and subsequent changes in the proliferative and apoptotic indices of these cells, as well as premature

lobuloalveolar development and milk protein production in virgin female mice (Sympson *et al*, 1994; Witty *et al*, 1995b). STR-1 is normally expressed during murine mammary gland development, however its expression is confined to stromal cells surrounding the developing ducts (Witty *et al*, 1995b). We have shown that MAT is endogenously expressed, albeit at very low levels, in the adult murine mammary gland by RT-PCR (Wilson *et al.*, 1995). Although we have been unable to localize MAT expression in this tissue by in situ hybridization or immunohistochemistry, studies with human mammary glands demonstrate that MAT mRNA and protein are expressed in mammary epithelial cells (Saarialho-Kere *et al*, 1995; Heppner *et al*, 1996). Examination of other murine tissues also suggests that MAT is primarily expressed in glandular epithelial cells (Wilson *et al*, 1995). Therefore, we were interested in investigating the effects of overexpressing the epithelium-specific MMP MAT in the epithelial cells of developing murine mammary glands and comparing the effect to previous results with STR-1 overexpression.

The ductal tree of developing mammary glands in nontransgenic and MMTV-MAT transgenic mice was examined by whole mount tissue preparation. During mammary gland development, which begins at approximately 5 weeks of age and following the onset of estrogen production, the mammary end buds grow outward from the nipple to fill the entire fat pad with a highly branched network of epithelial cells (Snedeker *et al*, 1991 and references therein). We show that there is no apparent morphological difference in the mammary ductal tree during any stage of development in virgin MMTV-MAT (Figure 5A and B) when compared to nontransgenic littermate controls (Figure 5C and D). We also observed no difference in mammary gland morphology in the MMTV-ActMAT and MMTV-InMAT animals (data not shown).

Although the MMTV-MAT glands display normal morphology, we examined them for subtle changes in differentiation, proliferation, or apoptosis that may have

occurred in response to MMTV-MAT. Production of the milk proteins in the casein family is normally restricted to differentiated mammary epithelial cells during late pregnancy and lactation. However, using an antibody specific for mixed caseins, milk proteins were detected in all virgin MAT transgenic animals previously shown to express the MAT transgene by northern analysis or MAT protein by immunohistochemistry (Figure 6A and B). No casein was detected in age-matched nontransgenic control mammary glands (Figure 6C and D). These results suggest that there is aberrant differentiation of mammary epithelial cells as a result of the transgene expression, although there are no accompanying morphological changes resembling lobuloalveolar development. The lack of morphological changes is consistent with our inability to detect differences in the number of proliferative or apoptotic cells in the MMTV-MAT mammary glands compared to age-matched, nontransgenic controls. We observed no significant difference in the number or location of proliferating cells as determined by immunoreactivity with the cell cycle marker PCNA, or apoptotic cells as determined by the number of cells with excessive nuclear DNA fragmentation (TUNEL assay; data not shown).

Localization of MAT Transgene Expression in the Male Reproductive Tract

The MMTV-LTR targets expression of a reporter gene to the male reproductive tract (Choi *et al*, 1987; Ross *et al.*, 1990). Several transgenic animals previously generated using this promoter have reported transgene expression in the testis and epididymis (Witty *et al*, 1995b: Matsui *et al*, 1990). In agreement with this, we found that the MAT transgene was expressed in both the testis and epididymis of MMTV-MAT transgenic mice. More specifically, the human MAT mRNA was localized by in situ hybridization to the primary spermatocytes of the transgenic testis (Figure 7A and B). In contrast, the human MAT protein localizes by immunohistochemistry on frozen sections of the testis to the interstitial space surrounding the seminiferous

tubules (Figure 7C and D). In the transgenic epididymis, the human MAT protein was also localized by immunohistochemistry to the epithelial ducts of the initial segment of the epididymis (Figure 8A and B). Endogenous murine MAT protein has been previously shown to be expressed in the epithelial cells lining the efferent ducts (Wilson *et al.*, 1995). The antibody that we have utilized for these studies is specific for human MAT as shown by the inability of this antibody to detect endogenous mouse MAT protein in the efferent ducts (Figure 8A and B and data not shown). In addition, the human MAT antibody detects the transgene product in the transgenic testis (Figure 7C and D) whereas the antibody specific for mouse MAT protein does not show specific staining in transgenic testis (data not shown).

Consequences of MAT Overexpression in the Male Reproductive Tract

When breeding the MMTV-MAT male transgenics, we noted that one of the male founder animals was infertile. In addition, other male founders and their offspring also demonstrated reduced fertility, producing few litters with only 1 to 3 pups. These same male transgenic animals eventually became infertile at approximately 6 months of age. We therefore analyzed several male transgenic gonads to address the cause of the observed reproductive defect. Hematoxylin and eosin stained sections of testis from 8 month old or older transgenic males show extreme disorganization of seminiferous tubule morphology (Figure 7F for example) compared to an age-matched nontransgenic controls (Figure 7E). In addition, an absence or reduced number of mature spermatozoa is noted in the seminiferous tubules of transgenic testis (Figure 7F) compared to the abundant presence of spermatozoa in the lumen of non-transgenic testis (Figure 7E). Less severe morphological disruption was also observed in younger males (data not shown). The MMTV-MAT transgenic epididymis demonstrated a lack of mature sperm production (Figure 8D) and the abnormal presence of sloughed undifferentiated germ cells was observed at higher

magnification (Figure 8F), compared to age-matched nontransgenic controls (Figure 8C and E). The morphology of the epididymis, and specifically the epithelial cells of the initial segment, appear normal (Figure 8), indicating that this is a local response and not a general effect on the male reproductive tract. Consistent with this observation, circulating androgen levels in aged transgenic male animals was not significantly different from age-matched non-transgenic controls (data not shown). These histological data support our initial observations of decreased fertility in the MMTV-MAT male transgenic animals. To our knowledge, perturbation of male reproductive function by MMP expression has not been previously observed.

Expression of the MAT Transgene in Other Organs

The MMTV-LTR is known to be expressed in the epithelial cells of the salivary glands, lungs, kidneys, and lymphoid cells of the spleen and thymus in addition to the tissues discussed above (Ross *et al*, 1990). We detected human MAT mRNA in the adult brain, salivary glands, lung, spleen, and thymus of MMTV-MAT transgenic mice, while the female reproductive tract and liver contained no detectable human MAT RNA (data not shown). Although these additional organs expressed MAT, there were no observable functional or morphological changes in these tissues as a result of transgene expression (data not shown).

DISCUSSION

Our goal in this study was to generate transgenic animals that overexpress three separate forms of human MAT in the reproductive tissues. To this end, we first developed wildtype, active and inactive human MAT constructs and examined the ability of these constructs to produce functional MAT protein in an in vitro system. We have shown that full length wildtype human MAT cDNA under the control of

the MMTV-LTR promoter/enhancer was capable of producing full length pro-MAT that was efficiently converted to active MAT after exposure to an exogenous activator. A valine to glycine substitution in the highly conserved pro-domain sequence PRCGVPDV of MAT encoded a protein with an increased tendency to spontaneously generate active protein, similar to that previously observed with STR-1 (Sanchez-Lopez *et al*, 1988; Park *et al*, 1991). In addition, a glutamic acid to glutamine substitution in the conserved zinc-binding sequence of MAT resulted in a catalytically-inactive protein, as observed for STR-1 (Sanchez-Lopez *et al*, 1988; Park *et al*, 1991). Although we could detect MAT protein produced by these constructs in the conditioned medium of cultured breast carcinoma cells, mutations of the MAT cDNA significantly decreased the efficiency with which MAT protein was produced in the transgenic mammary gland in vivo. The relative mRNA and protein levels of MAT were reduced in animals expressing the active MAT construct, and we observed no intact mRNA and no immunostaining for MAT protein in animals containing the inactive MAT transgene (Figures 3 and 4). It was noted that mutations in the STR-1 protein reduces the efficiency of protein production (Sanchez-Lopez *et al*; 1988; Park *et al*, 1991). It was speculated that this was due to the presence of active enzyme in an intracellular compartment resulting in premature auto-degradation (Park *et al* , 1991), or possibly a conformational change which may have altered the secretory pathway of the mutant protein. Our data suggests that the inactivating mutation also results in an alteration in mRNA stability in vivo which contributes to inefficient expression.

The expression of wildtype MAT results in an altered phenotype in the mammary gland and male reproductive tract compared to nontransgenic controls. We assume this is the result of degradation of MAT substrates, although we were unable to definitively attribute the effect to MAT-induced proteolysis since the inactive MAT transgenic mice expressed no detectable MAT protein. However,

since MAT is the "minimal domain MMP", it is unlikely to possess biological activities other than proteolysis. Since phenotypic alterations were observed in the mammary gland and testis, wildtype MAT is apparently activated in the tissues. Similar endogenous activation of interstitial collagenase was assumed in transgenic mice expressing a human MMP-1 genomic fragment under the control of the haptoglobin promoter (D'Armiento *et al*, 1992). MMPs such as MAT are activated by the "cysteine switch" mechanism, in which the cysteine in the conserved PRCGVDV sequence in the pro domain becomes dissociated from the catalytic Zn, resulting in a conformation change and autoproteolysis of the pro domain (Van Wart and Birkedal-Hansen, 1990; Springman *et al*, 1990). The activation of latent MMPs is affected by a variety of natural molecules. For example, plasmin activates most MMPs by cleaving once in the pro domain producing an unstable intermediate form of the enzyme which then autoproteolyses to produce a fully active enzyme (Suzuki *et al.*, 1990; Nagase *et al.*, 1990; Eeckhout and Vaes, 1977; Santibanez and Martinez, 1993). Other enzymes such as cathepsin G, neutrophil elastase, trypsin, chymotrypsin, and plasma kallikrein, have also been shown to activate latent MMPs by similar mechanisms (Saari *et al.*, 1990; Grant *et al.*, 1987; Nagase *et al.*, 1990; Okada and Nakanishi, 1989). In addition to proteases, oxygen radicals are also potential activators of MMPs due to their ability to disrupt the cysteine switch (Burkhardt *et al*, 1986; Rajagopalan *et al*, 1996). At this time, the endogenous activator of MAT is unidentified, but it is interesting that phenotypic alterations are observed in some, but not all, tissues that express the wildtype MAT transgene. A more thorough examination of tissues in the MMTV-ActMAT mice may provide insights as to whether the absence of phenotypic differences is due to the lack of an effect of MAT in these organs, or the deficiency of an endogenous activator of latent MAT.

The Effect of MAT Overexpression on Mammary Gland Development

The developing murine mammary gland has provided an excellent model system to examine the role of MMPs in a remodeling tissue. The expression patterns of MMPs suggest that they play an important role in the dramatic morphological and functional changes that take place in the mammary gland during ductal development. Overexpression of human MAT protein had no effect on the general morphological development of the mammary ductal tree, but induced the ectopic expression of a pregnancy-associated protein, β-casein, in developing virgin transgenic mammary glands. In contrast, the MMTV-STR-1 (Witty *et al.*, 1995b) and WAP-STR-1 (Sympson *et al.*, 1994) transgenic animals express β-casein mRNA, but not protein, display the morphological features of precocious lobuloalveolar development, and demonstrate increased proliferation and apoptotic indices (Witty *et al*, 1995b; Boudreau *et al*, 1995). There are several potential explanations for these differences. Experimental variation, such as differences in the integration sites, expression levels, and genetic backgrounds of the mice may be contributing factors, although the phenotypes were observed in several independent lines of mice in all cases. STR-1 contains a hemopexin/vitronectin-like domain which is absent in MAT, and may confer additional activities or alter substrate specificity in vivo resulting in the observed phenotypic differences. The abnormal tissue-type expression of STR-1 in glandular epithelial cells, as opposed to the normal expression in stromal fibroblast-like cells surrounding the developing ducts (Witty *et al*, 1995b) may also account for the more profound cellular alterations in these mice compared to MAT transgenic animals. In addition, the differential endogenous expression levels of STR-1 and MAT in the mammary gland suggest that these MMPs may have distinct roles during mammary development. The low endogenous expression levels of MAT (Wilson *et al*, 1995) implies that this particular MMP plays a minor role in mammay development compared to the

abundantly expressed STR-1 (Witty *et al*, 1995b), which may explain the less dramatic consequences of MAT overexpression. Although the morphological features of lobuloalveolar development were not observed in the MMTV-MAT transgenic mice, they displayed features of lactational differentiation by the production of β-casein protein in virgin transgenic mammary glands. This implies that β-casein expression can be dissociated from the morphological changes, and may be directly related to alterations in the integrity of the basement membrane of mammary epithelial cells.

MMTV-MAT Expression in Male Reproductive Tract Induces Infertility

An unexpected consequence of generating MAT transgenic animals under the control of the MMTV promoter/enhancer was the development of abnormalities of the male reproductive tract, since this phenotype was not observed in the MMTV-STR-1 mice (Witty *et al*, 1995b). In the testis, spermatozoa normally develop within the seminiferous tubules in close association with the Sertoli cells, while androgens are synthesized between the tubules in the Leydig cells (reviewed in Johnson and Everitt, 1995). These two compartments are separated by structural and physiological barriers that develop during puberty prior to the initiation of spermatogenesis. The barriers consist of gap and tight junctional complexes that completely encircle each Sertoli cell, linking it to the next adjacent cell. A few molecules may traverse these junctional complexes and penetrate into the basal compartment of the tubules from the surrounding interstitium, usually as the result of selective transport. Ions and proteins not only flow from the Leydig cells into the tubules, but proteins such as androgen binding protein, testicular transferrin, and sulphated glycoproteins 1 and 2 move from the intratubular compartment out into the interstitial area surrounding the tubules (Gunsalus and Bardin, 1991; Griswold, 1988). Similar to these proteins, the mRNA and protein localization patterns of the

MAT transgene suggest that MAT protein is produced by the germ cells of the seminiferous tubules and selectively transported into the interstitial environment between the tubules. The protein localization of the MAT transgene in the testis suggests that its overproduction may result in the degradation of cellular barriers between the interstitium and the seminiferous tubules, and result in disorganization of the testis morphology. A functional consequence of this would be to disrupt sperm production, which is consistent with the absence of mature spermatozoa in the epididymis of these transgenic male animals. The degradative effects of MAT seem to be gradual, suggesting that a threshold of excess enzyme needs to be reached before damaging effects occur, or that the destructive effects are cumulative. The absence of mature spermatozoa in the transgenic epididymis may also be caused by the overexpression of MAT in the initial segment of the epididymis. Overexpression of the MAT transgene in the epididymis may have specific effects on sperm maturation by disrupting the spatial or temporal cleavage of specific substrates, or may have more nonspecific effects caused by excessive degradation of proteins in these organs.

High levels of endogenous MAT expression have been localized to the efferent ducts while low levels of endogenous MAT hybridization were also observed in the proximal area of the initial segment of the epididymis and in the cauda, where mature fully differentiated sperm accumulate in the lumen (Wilson *et al.*, 1995). Very little is know about the endogenous expression of other metalloproteinases and their inhibitors in the male reproductive tract. GEL A is expressed by cultured rat Sertoli cells (Sang *et al*, 1990a, b), and TIMP-1 and TIMP-2 have been detected in the Sertoli cells of maturing rats (Ulisse *et al*, 1994). In addition, precursor regions in the α and β subunits of the fertilin complex or PH-30, a sperm surface protein that has been implicated in sperm-egg fusion, contain metalloproteinase domains that align with those found in snake venom proteins

(Wolfsberg *et al*, 1993). The function of MAT and other MMPs in the male reproductive system is not known. However, the specific tissue expression pattern of endogenous MAT is suggestive of a role in sperm maturation, possibly by proteolytically processing sperm antigens. MAT-deficient and STR-1-deficient mice display no obvious defects in male fertility (Wilson *et al*, 1997 and our unpublished observations), although it is possible that other MMPs may compensate for the lack of these MMPs. In fact, we have observed upregulation of STR-1 and STR-2 in the involuting uterus of MAT-deficient mice, and a similar apparent compensatory mechanism in STR-1-deficient mice, suggesting that there is strong selective pressure for MMP activity in reproductive processes (Rudolph-Owen *et al*, 1997).

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FIGURE LEGENDS

Figure 1. A) MMTV-MAT transgenic construct. Diagram of the plasmid construct utilized for creation of transgenics. The filled boxes correspond to the 3′ slice sites and 5′ polyadenylation signals of the rabbit β-globin gene. Three forms (wildtype, active, and inactive) of the human MAT cDNA was inserted into the third exon of the β-globin gene. B) Mutations in the human MAT transgenic constructs. Wildtype, active, and inactive MAT cDNA sequence and corresponding amino acids are depicted surrounding the mutation sites. Boxed areas indicate the position of the nucleotide mutation and amino acid substitution. Active MAT contains a substitution in the pro domain at amino acid 92 from valine to glycine, while inactive MAT contains a substitution in the catalytic domain at amino acid 216 from glutamic acid to glutamine. C) Immunoprecipitation of the MAT protein. The breast cancer cell line Hs578t was transiently transfected with each MMTV-MAT construct, and the MAT protein immunoprecipitated from the conditioned medium as indicated by the arrows. 1μM of dexamethasone (Dex) was added to the culture medium of samples shown in lanes B, D, and F. APMA, a known MMP activator, was added to the conditioned medium of samples shown in lanes G, H, and I.

Figure 2. MMTV-MAT transgenic lines. Southern hybridization of 10μg of genomic DNA from wildtype founder animals (lines 1, 3, 24, and 42), active founder animals (lines 1, 5, and 22) and inactive founder animals (lines 2 and 4). The approximate copy number of each line is indicated below the lane.

Figure 3. Expression of the human MAT transgene in developing mammary tissue. Northern analysis of poly A+ selected RNA (4μg) from nontransgenic and transgenic female mammary tissue at various weeks during mammary development. Blots were probed with a 1.1kb ^{32}P-labelled human MAT cDNA probe (HMAT) and a

cyclophilin (1B15) cDNA probe was used to control for RNA loading. A) Expression of MAT transgene is present at relatively high levels in selected samples from line #3 and #42 and absent in the nontransgenic littermates. B) Line #1 and line #22 express the MMTV-ActMAT transgene, while nontransgenic littermates do not express the transgene. C) Isolation of intact human MAT mRNA from both MMTV-InMAT transgenic lines #2 and #4 was not possible after several attempts. Degraded human MAT mRNA as shown was consistently isolated in both lines of transgenic mammary glands.

Figure 4. Localization of human MAT protein by immunohistochemistry. Representative sections from developing transgenic (A, B, C) and nontransgenic (D) mammary glands were probed with an affinity-purified polyclonal antibody against a human MAT peptide. Staining of human MAT is in the cytoplasm of the mammary ducts from MMTV- MAT wildtype line #3 (A) and to a lesser extent in MMTV-ActMAT line #22 (B) developing glands (arrows). No specific staining was detected in MMTV-InMAT line #2 transgenic (C) or nontransgenic (D) mammary glands. Magnification = 50X.

Figure 5. Morphological appearance of developing mammary glands. Iron hematoxylin stained whole mounts of inguinal mammary glands from MMTV-MAT line #3 transgenic (A and B) and nontransgenic (C and D) female virgin animals. Glands were removed at 14 weeks (A and C) and 16 weeks (B and D) of mammary gland development. Magnification = 2.5X (A and C) and 6.4X (B and D).

Figure 6. Casein expression in virgin MMTV-MAT mammary glands. Wildtype MMTV-MAT line #3 transgenic mammary glands (A and B) at 6 weeks of age were positive for β-casein expression using immunohistochemistry, while nontransgenic

control mammary glands (C and D) were negative. Panels A and C were taken using 16X magnification and panels B and D were taken using 32X magnification.

Figure 7. Expression and consequence of human MAT in the testis. Sections from the testis of an adult wildtype MMTV-MAT line #3 male were hybridized with an antisense ^{35}S-labelled human MAT probe (A, dark-field and B, light-field; 40X). Hybridization was localized to the primary spermatocytes (arrows) in the adult testis (B). Frozen sections of the testis from a wildtype MMTV-MAT line #3 transgenic male were probed with a polyclonal antibody specific to human MAT. MAT transgene product is localized to the interstitial space surrounding the seminiferous tubules as can be seen at low power (C; 12.5X) and at higher power (D; 32X). Hematoxylin and eosin staining of sections obtained from normal (E) or MMTV-MAT wildtype line #3 transgenic (F) adult testis (both at 12.5X magnification).

Figure 8. Expression and consequence of human MAT in the epididymis. Frozen sections of the epididymis from a wildtype MMTV-MAT line #3 transgenic male were probed with a polyclonal antibody specific to human MAT. The protein is localized to the epithelial ducts of the upper-most region of the epididymis, the initial segment (in. seg.), as can be seen at low power (A; 6.25X magnification; distal caput, dis. cap.; efferent ducts, e. d.) and high power (B; 12.5X magnification). Hematoxylin and eosin staining of normal (C and E) or wildtype MMTV-MAT line #3 transgenic (D and F) adult epididymis. Magnification = 16X (C and D) and 50X (E and F).

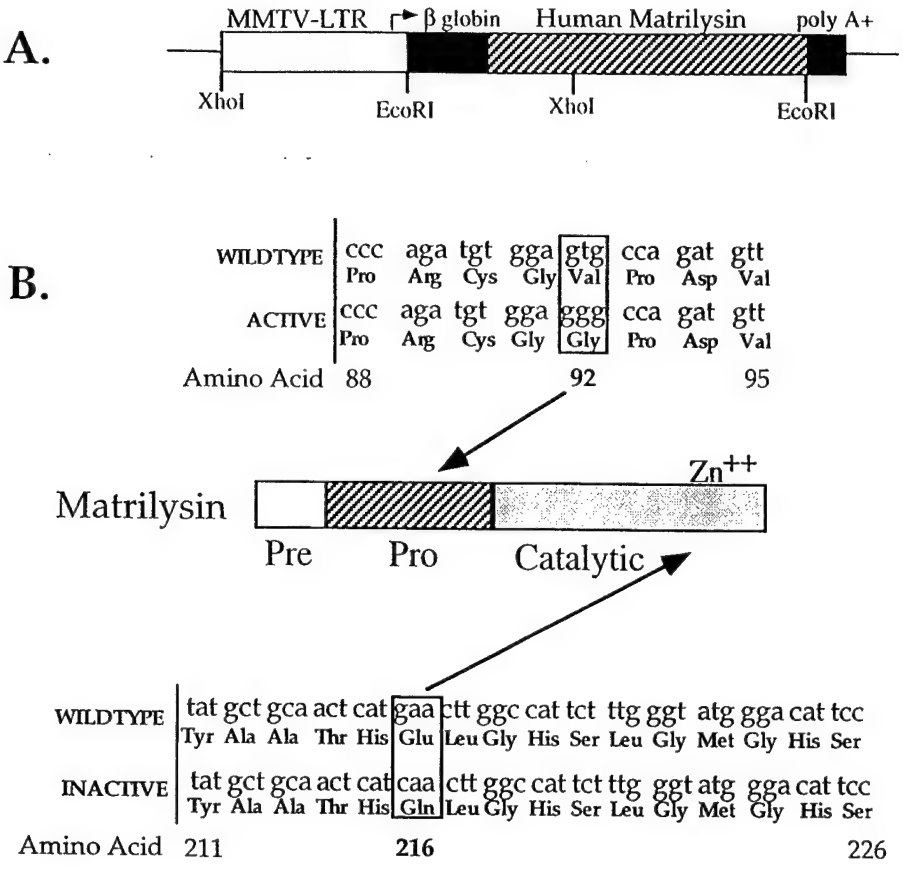
A.
MMTV-LTR
β globin
Human Matrilysin
poly A+
XhoI
EcoRI
XhoI
EcoRI
B.
WILDTYPE
ccc aga tgt gga gtg cca gat gtt
Pro Arg Cys Gly Val Pro Asp Val
ACTIVE
ccc aga tgt gga ggg cca gat gtt
Pro Arg Cys Gly Gly Pro Asp Val
Amino Acid 88 92 95
Matrilysin
Zn++
Pre
Pro
Catalytic
WILDTYPE
tat gct gca act cat gaa ctt ggc cat tct ttg ggt atg gga cat tcc
Tyr Ala Ala Thr His Glu Leu Gly His Ser Leu Gly Met Gly His Ser
INACTIVE
tat gct gca act cat caa ctt ggc cat tct ttg ggt atg gga cat tcc
Tyr Ala Ala Thr His Gln Leu Gly His Ser Leu Gly Met Gly His Ser
Amino Acid 211 216 226
C.
Wildtype
Wildtype + Dex
Active
Active + Dex
Inactive
Inactive + Dex
Wildtype + APMA
Active + APMA
Inactive + APMA
46K
30K
21.5K
latent form
active form
A B C D E F G H I

Figure 1

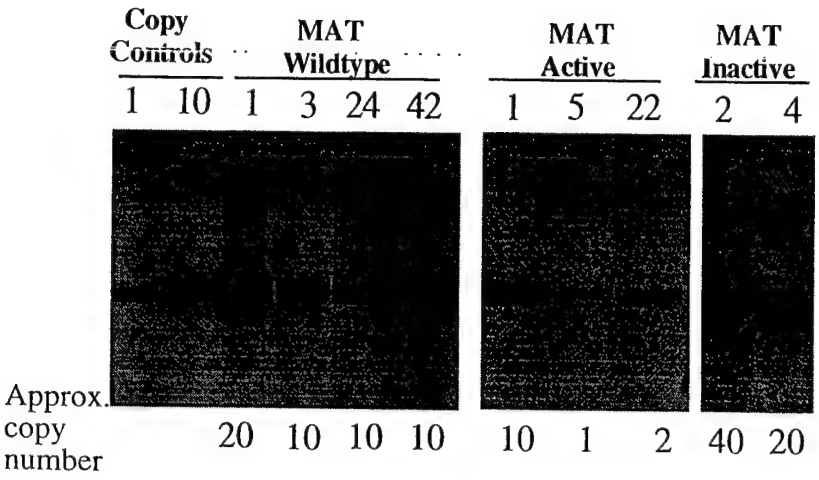
Copy Controls
MAT Wildtype
MAT Active
MAT Inactive
1 10 1 3 24 42 1 5 22 2 4
Approx. copy number
20 10 10 10 10 1 2 40 20

Figure 2

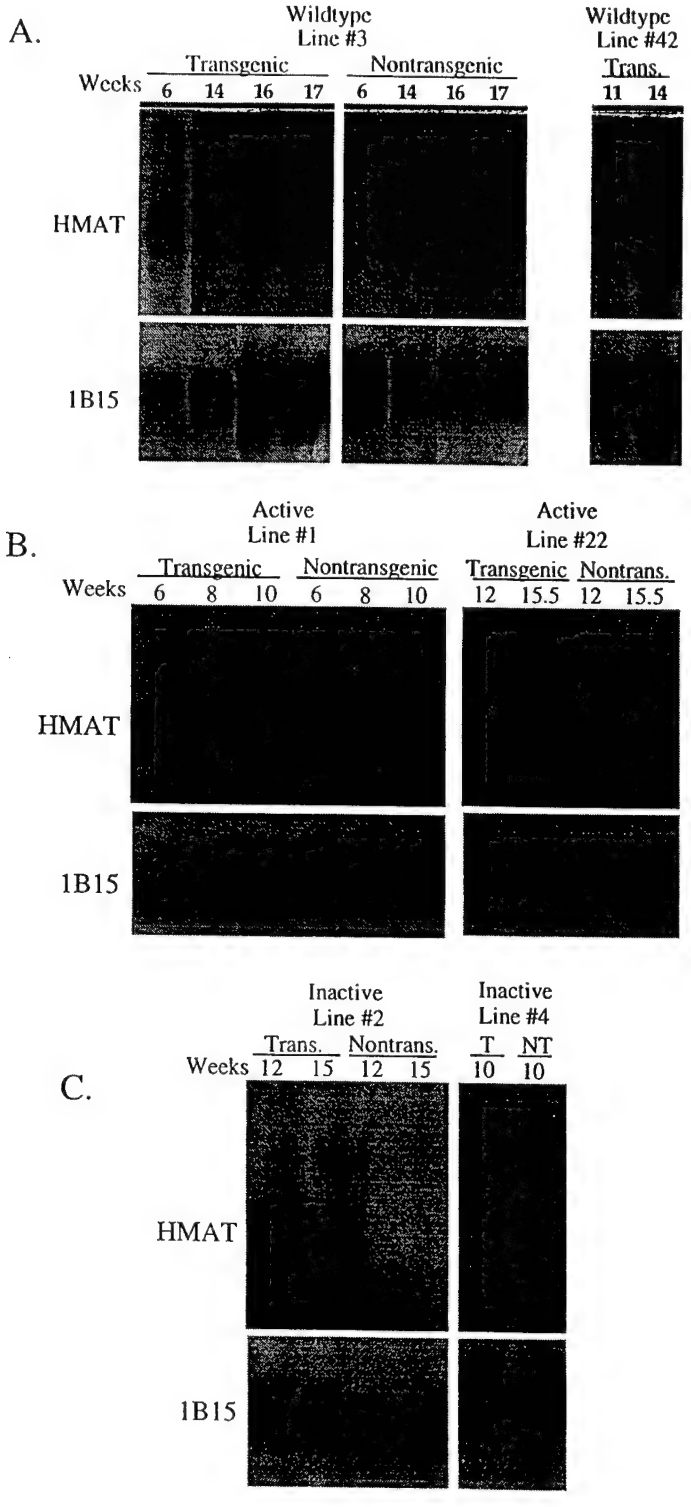
A.
Wildtype
Line #3
Transgenic
Nontransgenic
Weeks
6 14 16 17
6 14 16 17
Wildtype
Line #42
Trans.
11 14
HMAT
1B15
B.
Active
Line #1
Active
Line #22
Transgenic
Nontransgenic
Transgenic
Nontrans.
Weeks
6 8 10
6 8 10
12 15.5 12 15.5
HMAT
1B15
C.
Inactive
Line #2
Inactive
Line #4
Trans.
Nontrans.
T
NT
Weeks
12 15 12 15
10 10
HMAT
1B15

Figure 3

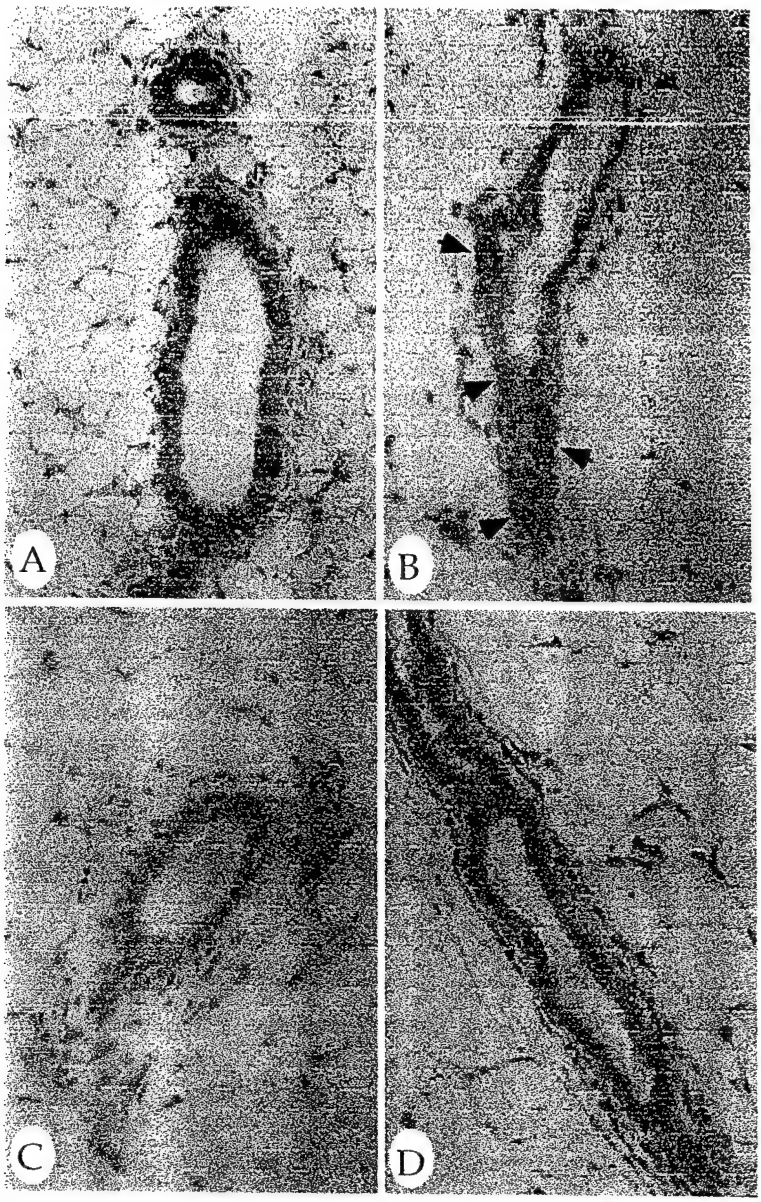

Figure 4

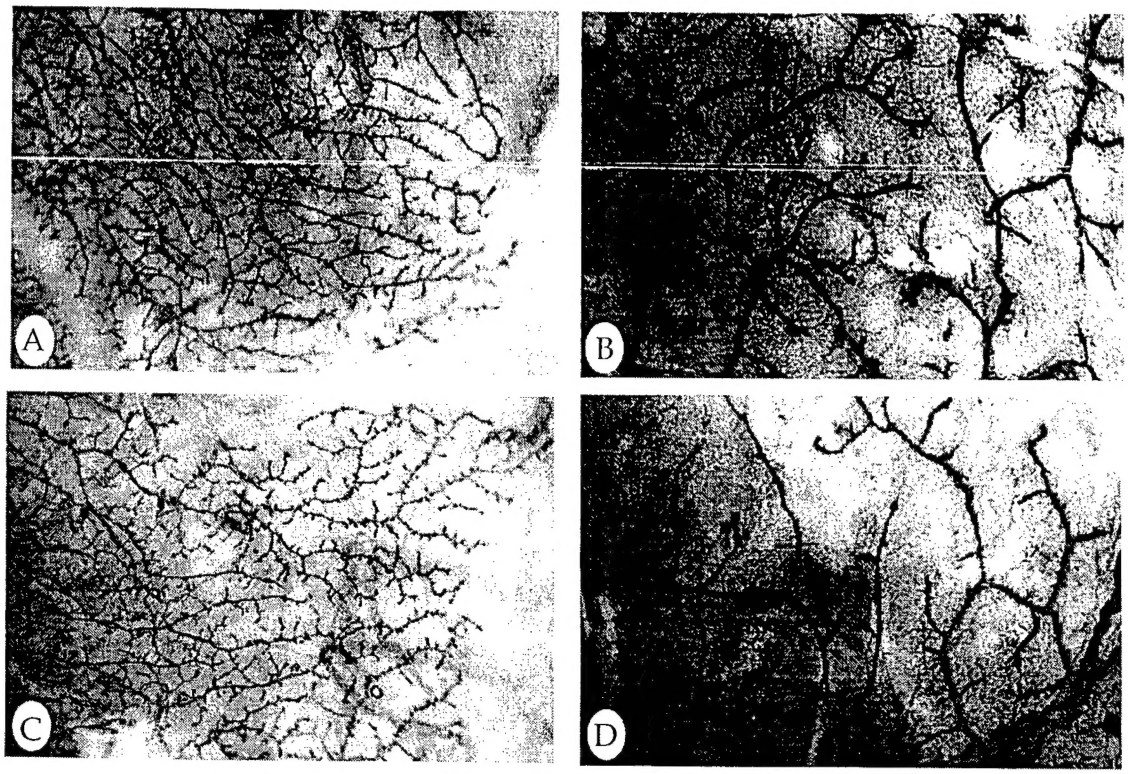

Figure 5

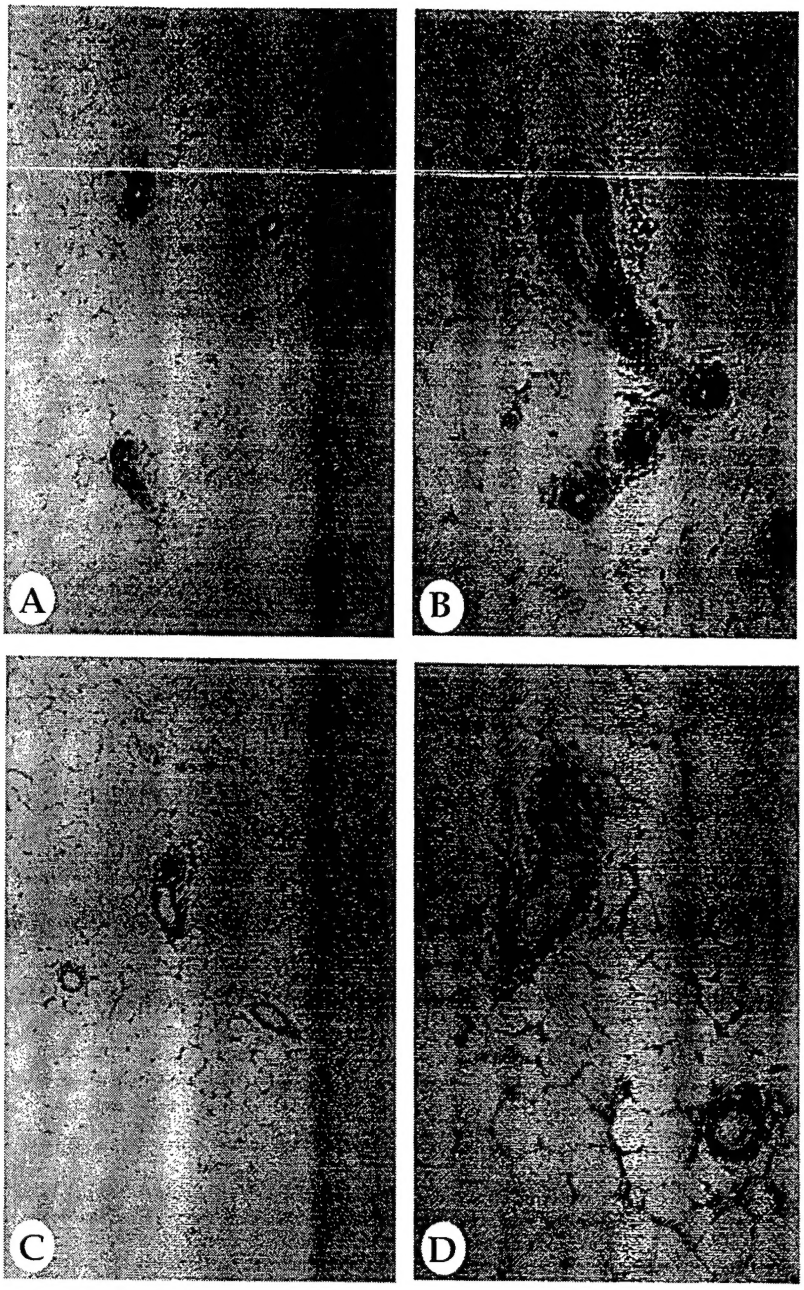

Figure 6

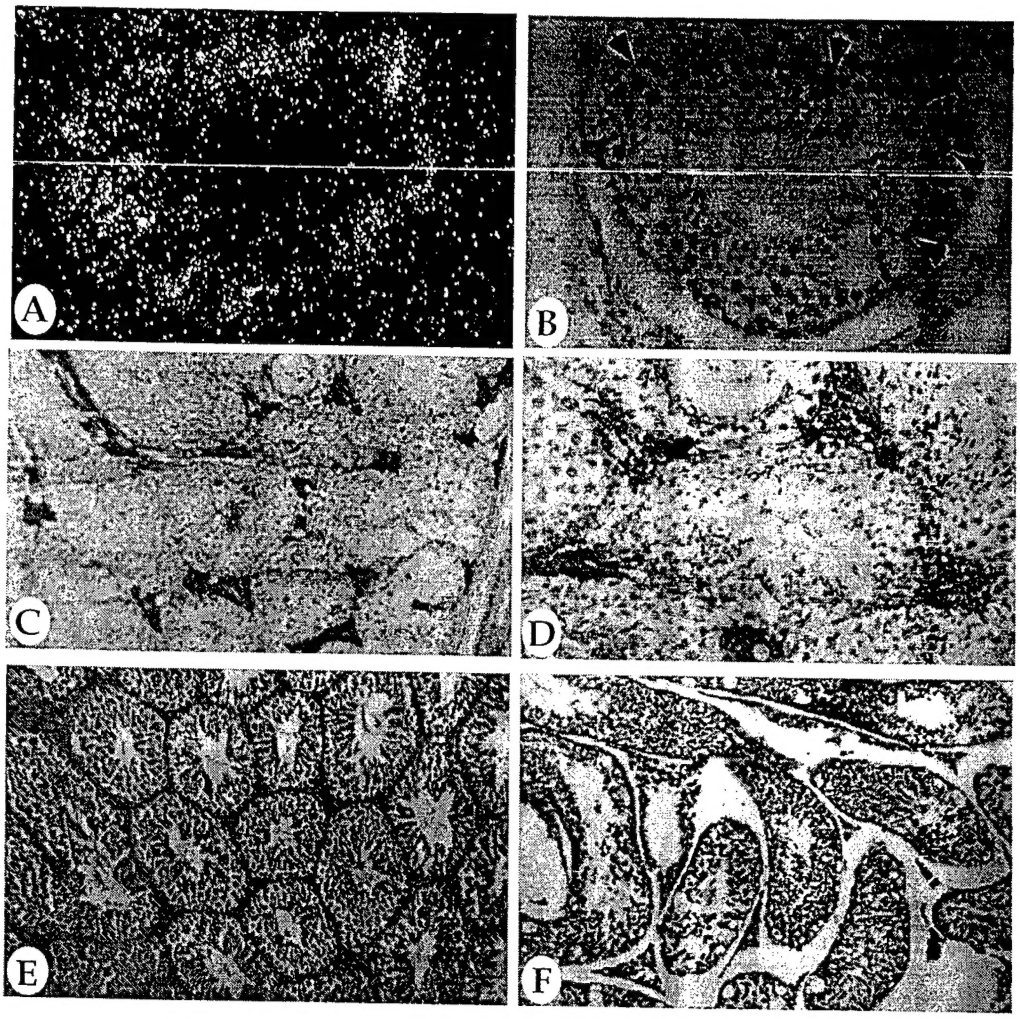

Figure 7

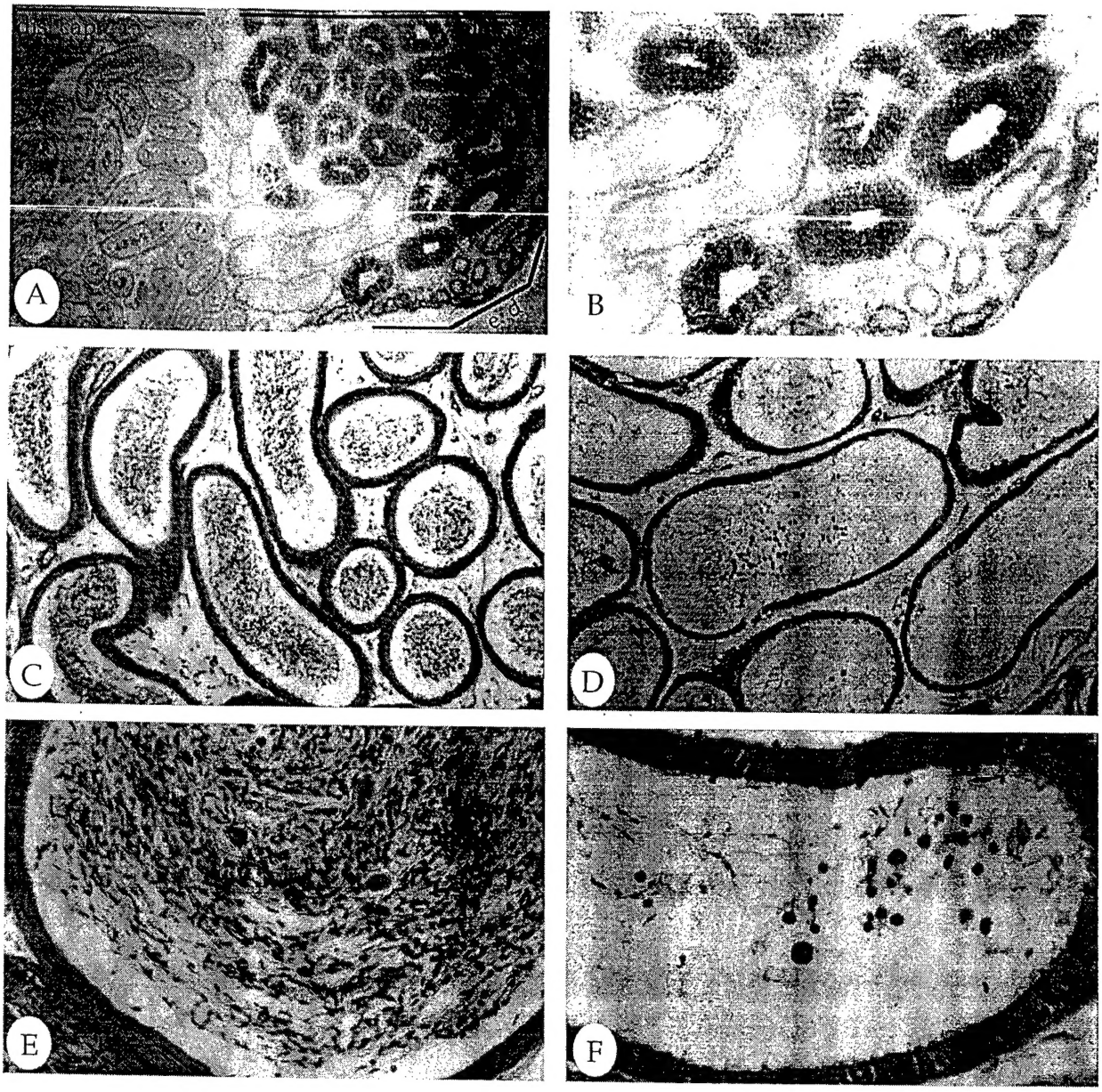

Figure 8